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Apoptosis in relation to neuronal loss in experimental Creutzfeldt-Jakob disease in mice

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Abstract. Apoptosis constitutes a genetically determined process to eliminate superfluous or damaged cells in tissues. Deficiencies in apoptosis regulation are involved in different pathologies including prion diseases. Some experimental studies show that neuronal loss - one of the hallmarks of prion diseases may be accomplished by apoptosis. We evaluated twenty five mice infected experimentally with the Fujisaki strains of CJD and sacrified sequentially in one week intervals. Apoptotic cells in various brain regions were detected by *in situ* end labelling (TUNEL) and electron microscopy in comparison with neuronal cell loss. The number of labelled cells per brain was very low - from a few labelled cells 6 weeks after inoculation to a maximum of 14 in the terminal stage. The number of neurones counted in 8 selected areas were considerably lower in terminally sick animals (20 and 21 week of incubation period) than in control mice. The mean value of loss of neuronal cells was 32%. The greatest loss (55%) of neurones was noted in the septal nuclei of the paraterminal body and the least lost (16%) in the hypothalamus. Compared to the extensive neuronal loss (30-50%), the number of apoptotic cells detected by *in situ* end labelling seems to be very low, and the process of neuronal death become more intensive during the progression of the disease.

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INTRODUCTION

Creutzfeldt-Jakob disease (CJD) is a rapidly progressive dementia caused by accumulation of an abnormal isoform of a host-encoded glycoprotein, termed prion protein (PrP); CJD belongs to transmissible spongiform encephalopathies (TSEs), or prion diseases. Despite the phenotypic variation of neuropathological changes in sporadic CJD, four classical neuropathological hallmarks such as spongiform change, astrocytosis, neuronal loss and amyloid deposits have been discriminated (Brown et al. 1994, Ironside 1996, Mikol 1996) - neuronal cell loss may be accomplished by apoptosis. There is much evidence that apoptosis contributes to the neuronal loss in TSEs (Giese et al. 1995, Lucassen et al. 1995, Fraser et al. 1996, Kretzschmar et al. 1996, 1997, Robin et al. 1996, Lucas et al. 1997). The neurotoxicity of PrP^{Sc} and its internal PrP (106-126) peptide has been demonstrated experimentally *in vitro* (Müller et al. 1993, Brown et al. 1994). Moreover, a recent study has shown that the viability of cultured rat neurones is reduced in a time- and dose-dependent manner following addition of PrP (106-126) (Forloni et al. 1996, Perovic et al. 1997). Coculture experiments showed that PrP (106-126) neurotoxicity is mediated by microglial cells activated upon exposure to this PrP peptide (Brown et al. 1996, Kretzschmar et al. 1996). Müller et al. demonstrated that PrP^{Sc} decreased the viability of rat cortical cells and induced the DNA fragmentation and cell death in a dose and time-dependent manner; in contrast, astrocytes treated by PrP^{Sc} do not undergo cell death (Müller et al. 1993). Furthermore, the analysis of chromatin structure revealed DNA fragmentation into nucleosomal sized fragments in the cerebral cortex of a patient with CJD, but not in peripheral blood lymphocytes (Lucas et al. 1997). In contrast, hamsters inoculated with the 139A-H scrapie strain developed cellular death in the islets of Langerhans, due to necrosis but not apoptosis (Ye et al. 1997). The absence of amyloidal deposits and extremely low PrP^{Sc} levels in these peripheral organs suggests that the toxic effect of PrP^{Sc} resulting in apoptosis is specific for brain tissues, and that changes in the pancreatic islets may be merely secondary to scrapie-induced neurodegeneration of the hypothalamic neurones.

Since apoptosis involves degradation of DNA into 3'OH oligonucleosome-lenght fragments, it is readily detectable by *in situ* end labelling on paraffin sections (Lucassen et al. 1995, Fraser et al. 1996, Kordek et al. 1996, Robin et al. 1996, Jesionek-Kupnicka et al. 1997, Kretzschmar et al. 1997, Williams et al. 1997, Ferrer 1999). At the ultrastructural level, the characteristic features of apoptosis in cells such as chromatin condensation, nuclear fragmentation, electron dense cytoplasm, membrane blebbing and apoptotic cells have been demonstrated (Williams et al. 1997). The aim of our study was a demonstration of apoptotic cells in paraffin section of mouse brains with experimental CJD as a function of time of post-incubation period. Then we estimated the number of neurones in terminally sick animals and in control mice. We report here that apoptotic cells are readily detectable in CJD-affected mouse brains in time-depended manner after infection of Fujisaki strain, but the number of apoptotic cells detected by *in situ* end labelling does not correlate well with the extensiveness of neuronal loss.

METHODS

Material

Brain tissues were obtained at weekly intervals from 25 mice, aged 4-6 weeks, infected intracerebrally with the 0.03 ml of 10% clarified suspension of brain tissue (3×10^4 LD_{50}) of Fujisaki strain of CJD. Four mice were inoculated with normal mouse brain suspension and served as negative controls. The mice brains were fixed in 10% formalin, pH 7.0, than incubated in formic acid for 1 h, subsequently washed in distilled water and again immersed in formalin and embedded in paraffin. The mice were killed sequentially at one-week intervals, and the control mice were killed at the end of the experiment.

Four transverse paraffin sections, 5 μm thick, were cut from each brain and were stained with haematoxylin and eosin. Each brain section was than investigated for apoptosis and neuronal loss.

Cell death assay

To detect apoptotic cells, we used *in situ* end-labelling of 3'-OH oligonucleosome-length DNA fragments with modified TUNEL assay (ApopTaq In Situ Apoptosis Detection Kit Oncor, Gaithersburg MD, USA). This method is briefly described as follows: the deparaffinized and rehydrated 5 μm sections were digested with proteinaze K (Sigma) (120 μg/ml) for 20 min at room temperature. The slides were than washed in distilled water and immersed in 2% H_20_2 in distilled water for 10 min to block the activity of endogenous peroxidase. Af-

ter the wash in phosphate buffered saline pH 7.4 (PBS) (Sigma) the slides were incubated in equilibration buffer for 10 min at room temperature. Than, we applied 30 µl of a mixture containing terminal deoxynucleotidyl transferase (TdT) and the reaction buffer containing dATP and digoxigenin-11-dUTP. The sections were placed in a humified chamber at 37°C in a closed box for 1 h. After that the sections were immersed in stop/wash buffer for 10 min at room temperature and washed in PBS. The sections were incubated with anti-digoxigenin-peroxidase for 30 min at room temperature. After washing in PBS the slides were stained by 3.3 diaminobenzidine with 0.1% H_20_2 for 5-7 min. Sections were stained with haematoxylin, hydratated, and mounted with glue. Control sections were prepared in parallel with substitution of distilled water instead of TdT enzyme.

Quantitation of apoptotic nuclei

The numbers of apoptotic cells were enumerated in all four transverse paraffin brain sections of CJD-infected mice (Table II) and the control mice. The positive reaction for apoptosis was considered when there was brown reaction product, but the striking morphologic changes as nuclear condensation or crumbling, sometimes appearance of apoptotic bodies, shrinkage of the cytoplasm, with an absence of any underlying inflammatory component were also required. The rare artefacts appeared as homogenous deposits of brown pigment. Rare unspecific labelling of nonchanged nuclei was excluded as a positive reaction product.

Ultrastructural study

To confirm apoptosis in neurones, several fresh tissue samples were taken for electron microscopy. The tissue was perfusion-fixed in 1% paraformaldehyde 2,5% glutaraldehyde in cacodylate buffer, post fixed in osmium tetroxide, and embedded in Epon. Ultrastructural studies were performed with EM 109 Zeiss transmission electron microscope.

Estimation of neuronal loss

To estimate neuronal counts we used four control mice and four terminally sick mice sacrificed at 20th and 21st week post-inoculation. The terminally sick animals were defined as mice with fully developed neurological symptoms characteristic for CJD. In each brain we have chosen for neuron counting nine regions of grey matter in four transverse paraffin sections according to the lesion profile method developed by Fraser and Dickinson 1968, namely: the dorsal half of medulla including the cuneate, vestibular and cochlear nuclei, locus coeruleus, or the sensory nucleus of the trigeminal nerve depending on the exact levels available (1); the cerebellar cortex of the folia adjacent to the fourth ventricle, particularly the lingula or nodulus (2); the cortex of the superior colliculus (3); the hypothalamus (4); the thalamus, in particular in the massa intermedia and central nuclei of the thalamus (5); the hippocampus (6); the septal nuclei of the paraterminal body (7); an area of the cerebral cortex at the level of 4 and 5 lying dorsal to the corpus callosum, associated with the indusium griseum, the cingulate gyrus and cortex extending onto the dorsal surface of the brain (8); a similar area of cerebral cortex as in 8, associated with the section on which the septal nuclei are situated (9).

The number of neurones in hippocampus was not counted due to technical damage.

The number of neurones was estimated simultaneously by two of us (J.B. and D.J.K.) in five fields (magnification 1,000 x) in eight regions. Specimens were coded and the examiners were blinded to the affected vs. control animals when counting. Then we calculated the average number of neurones in given region.

Identification of neurones *vs.* glial cells was performed on the basis of nuclear morphology.

RESULTS

In CJD-infected mice brains, we visualised apoptotic cells as early as at the 6th week post- inoculation. The positive reaction was present as a brown reaction product in nuclei of cells displaying characteristic apoptotic morphology, i.e. nuclear condensation, shrinkage of the cytoplasm, appearance of apoptotic bodies with an absence of any underlying inflammatory component (Fig. 1). These features are very useful to discriminate between apoptosis and necrosis unspecified reaction. Consecutively, the number of ApopTaq-positive cells increased through the 13-th week post inoculation and there was a general trend toward increasing number of apoptotic cells (the range in weeks 6-13 was 1-3.5 cells, in weeks 14-18 was 2-7 cells, and in weeks 19-21 was 7.6-8 cells) (Table I). In control mice brains we detected no apoptotic cells.

By electron microscopy, some dark neurones with retracted membranes were occasionally observed; even less often, classic apoptotic neurones were seen (Fig. 2).

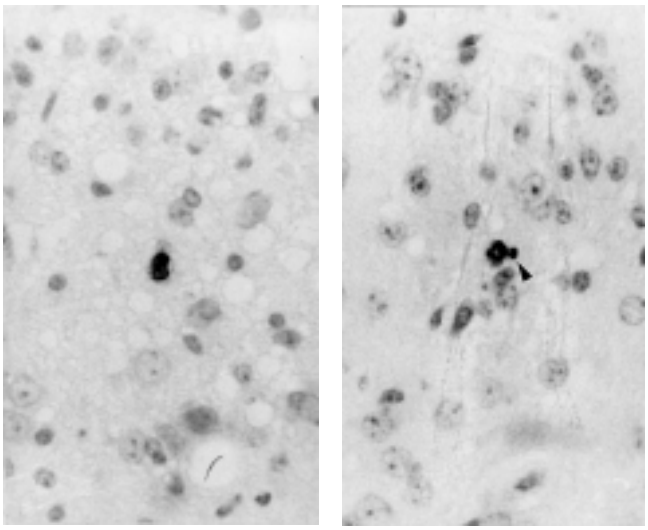

Fig. 1. Apoptotic cells in experimental CJD showing nuclear condensation, shrinkage of cytoplasm and apoptotic bodies (arrowhead). *In situ* DNA labeling, haematoxylin counterstain, x 400.

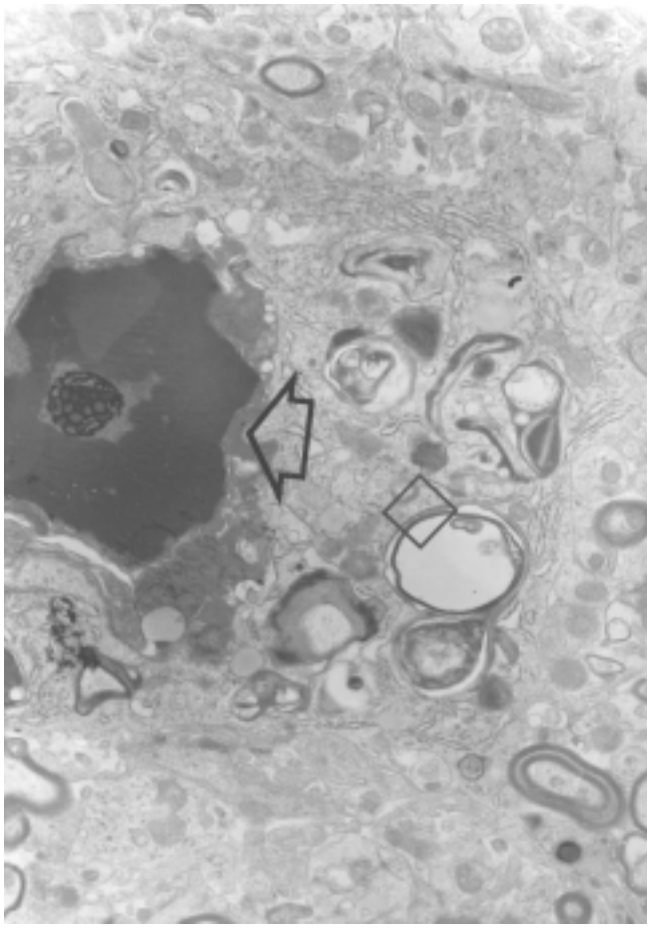

Fig. 2. An overview of the neuropathology of experimental CJD. Note an apoptotic neuron (arrow) with adjacent macrophage (square). Original magnification, x 5600.

Table I

The number of ApopTaq-positive cells in CJD-infected mice brains depending on the time post-inoculation

No. Case	Time post inoculation (weeks)	Number of apoptotic cells	Average number of apoptotic cells per week
1	4	0	0
2	5	0	0
3	6	1	1
4	7	1	1
5	8	1	1
6	10	1	1
7	12	3	
8	12	4	3.5
9	13	1	1
10	14	6	
11	14	4	5
12	15	2	2
13	16	10	
14	16	1	5.5
15	17	1	2.5
16	17	4	
17	18	2	2
18	18	2	7
19	19	7	
20	20	9	
21	20	1	
22	20	14	8
23	21	11	
24	21	5	
25	21	7	7.6

The number of neurones counted in eight selected regions of grey matter was considerably lower in the four terminal mice (20 and 21 week of post-inoculation) than in four control animals (Table II). The greatest loss of neurones (55%) was noted in the septal nuclei of paraterminal body (region 7) while the least loss (16%) was seen in the hypothalamus (region 4). The mean value of loss of neuronal cells was 32% (Table II, Fig. 3).

We attempted to examine both apoptosis and neuronal loss in the same regions of the transverse sections of brain. Unfortunately, the apoptotic cells were randomly distributed in different brain structures, and the low number of detected apoptotic cells did not allow to relate them to the given regions, because in the most areas the number of stained cells was zero.

Table II

The number of neurones estimated in eight selected areas of grey matter in four transverse paraffin brain sections of CJD-infected mice and of control mice

	Number of neurones								
	1	2	3	4	5	6	7	8	9
	the dorsal half of medulla	the cerebellar cortex	the cortex of the superior culliculus	the hypotha-lamus	the thalamus	the hippo-campus	the septal nuclei of paraterminal body	the cerebral cortex	the cerebral cortex*
Control mouse						NC			
1st	56.4	40.3	102.6	67.8	69.0	-	130.0	89.8	79.4
2nd	80.6	62.2	112.2	67.4	66.0	-	142.0	65.0	65.2
3rd	68.0	42.4	91.6	80.0	65.0	-	83.6	69.2	64.8
4th	68.2	50.2	63.0	68.0	59.8	-	65.6	100.0	90.8
Mean value of neurones in control mice									
	63.3	48.7	92.35	70.8	64.9	-	105.3	81.0	75.0
Terminally ill mouse (weeks post inoculation)						NC			
20 week	39.4	34.2	88.4	63.4	35.0	-	64.0	54.4	57.2
20 week	35.8	41.2	0	65.4	37.8	-	42.0	56.6	46.6
21 week	45.0	33.3	58.0	56.6	52.4	-	41.2	38.6	48.0
21 week	48.4	29.4	65.4	53.2	50.6	-	42.6	56.8	56.4
Mean value of neurones in ill mice									
	42.2	34.5	70.3	59.65	43.95	-	47.45	51.2	52.05
Loss of neurones (%)									
	33	29	24	16	33	-	55	37	30

NC, not counted; *see Methods for details.

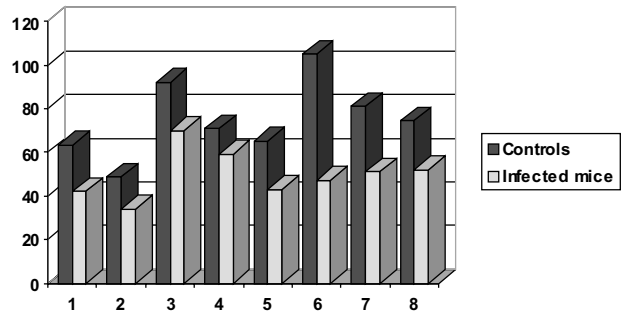

Fig. 3. Histogram representing the number of neurones in terminal and control mice.

DISCUSSION

Loss of neurones through programmed cell death is necessary during structural and functional maturation in the developing nervous system. More than half of all neurones born dies before they achieve functional maturity in the adult nervous system (Burek and Oppenheim 1996).

In experimental prion diseases, the neuronal cell loss in a given area of the brain depends on the PrP genotype, strain of prions, and site of inoculation. It has been demonstrated in various murine scrapie models, that loss of neurones develops in a precise neuroanatomical pattern dependent on a scrapie strain and mouse *Prn-p* genotype, and route of inoculation. Fraser et al. (1996) in intraocularly infected Me7 murine scrapie reported neuronal loss above 95% in dorsal lateral geniculate nucleus, the pyramidal cells of the hippocampus, and retinal photoreceptor cells. Following intracerebral inoculation, a major loss was seen in the CA1 hippocampal pyramidal cells. Jeffrey et al., reported that by the morphometric analysis a 50% loss of neurones of the vestibular nucleus occurred in bovine spongiform encephalopathy (Jeffrey et al. 1992).

In the present study, we demonstrated that apoptotic cell death increased consecutively in terminally sick mice in the time-dependent manner after inoculation and it may contribute to the neuronal loss in mice infected intracerebrally with the Fujisaki strain of CJD. Our work confirms the previous study in another model of experimental murine scrapie (79A strain), which demonstrated a presence of apoptosis and increase of TUNEL positive cells in the time course after inoculation (Giese et al. 1995). The highest numbers of labelled nuclei were found in the granular cell layer of the cerebellum of terminally ill mice and retina in the outer nuclear layer 120 days post infection followed by massive cell loss in this layer. In the present study, the most severe neuronal loss reached 55% in the septal nuclei of the paraterminal body (region 7), and the mean value of loss of neuronal cells approached 32%.

The number of neurones estimated in eight selected regions of grey matter was considerably lower in terminal (20-21 week of incubation period) than in control mice. In our study, the apoptotic cells were randomly distributed in different brain structures, and the low number of detected apoptotic cells did not allow to relate them to the number of neurones in given regions.

Apoptotic cells are not readily identified by light microscopy. Detection is hampered by the fact that cells undergoing apoptosis disappear within hours after nuclear DNA fragmentation is accomplished and therefore may be difficult to document in paraffin sections not to mention electron microscopy. Kretzschmar et al. (1996) observed that the most intensive cell death occurs not in terminally sick animals but earlier. It raises a possibility that cells dying early are more susceptible to programmed suicide while in the terminal stage of disease the remaining cell population is more resistant to scrapie. More importantly, the total number of neurones decreased throughout the incubation period. It is known that TUNEL method is not sufficient to detect the early phase of apoptosis because some dissociation from internucleosomal cleavage of DNA may exist (Cohen et al. 1992, Oberhammeret al. 1993). As a result, we may underestimate the apoptotic ratio in the terminal stage of the disease, compared to the pre-clinical phase. The interpretation of the apoptotic ratios requires further caution. TUNEL is a characteristic but not specific marker for apoptosis because DNA fragmentation can also be detected in necrosis or may occur artifactually in cells showing apparently normal morphology with no evidence of either apoptosis or necrosis (Enright et al. 1994). The cell death in CNS may thus be mediated by different mechanism. Not only apoptosis but necrosis and hybrid forms of cell death along an apoptosis-necrosis continnum have been described depending on models of brain injury and brain maturity (Portera-Cailliau et al.1997, Martin et al. 1998). Moreover, these events may temporally overlap and differ during progression of cell death under physiological and pathological conditions, and may be indistinguishable especially in the immature brain. For such a reason we considered as apoptotic only the cells with the striking morphologic changes, with an absence of any underlying inflammatory process. Unfortunately the type of apoptotic cells was not identified by double staining, but the significant decrease of the number of neurones in terminally sick animals may give an evidence that neuronal loss is accomplished by apoptosis.

In conclusion, it seems that in relation to a wide extensiveness of neuronal loss (30-50%) in experimental CJD in mice the number of apoptotic cells detected by *in situ* end labelling is underestimated. This method is very useful to reveal the apoptotic events *per se* in tissue section but is not sufficient to qualify an intensity and dynamics of this process. Due to limited usefulness of TUNEL method further investigations are necessary to resort to a specific marker of apoptosis, e.g. immunodetection of activated caspase-3 (Stadelmann et al.1999).

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